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Curcumin inhibits renal cyst formation and enlargement in vitro by regulating intracellular signaling pathways

Jinsheng Gao [1], Hong Zhou [1], Tianluo Lei, Li Zhou, Weidong Li, Xuejun Li, Baoxue Yang *

Department of Pharmacology, School of Basic Medical Sciences, Peking University, and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, 100191, China

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ABSTRACT

Autosomal dominant polycystic kidney disease, a common inherited disease affecting about 1/1000 and 1/400 live births, is characterized by massive enlargement of fluid-filled cysts and eventually causes renal failure. The purpose of this study is to identify the inhibitory effect of curcumin on renal cyst development and to investigate the inhibitory mechanism. Madin–Darby canine kidney (MDCK) cyst model and murine embryonic kidney cyst model were used to evaluate inhibitory activity. Cell viability, proliferation, apoptosis, CFTR function and expression, and signaling pathways in MDCK cells were determined to explore the mechanism of cyst inhibition. Curcumin was found to significantly inhibit MDCK cyst development. At maximum dose curcumin caused 62% inhibition of the cyst formation (IC_{50} was 0.12 μM). Curcumin slowed cyst enlargement in both MDCK cyst model and embryonic kidney cyst model with dose–response relationship. Curcumin neither induced cytotoxicity nor apoptosis in MDCK cells at <100 μM. Curcumin failed to affect the chloride transporter CFTR expression and function. Interestingly, curcumin inhibited forskolin-promoted cell proliferation and promoted the tubule formation in MDCK cells, which indicates curcumin promotes MDCK cell differentiation. Furthermore, curcumin reduced the intracellular signaling proteins Ras, B-raf, p-MEK, p-ERK, c-fos, Egr-1, but increased Raf-1 and NAB2 in MDCK cells exposed to forskolin. These results define that curcumin inhibits renal cyst formation and enlargement and suggest that curcumin might be developed as a candidate drug for polycystic kidney disease.

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1. Introduction

Autosomal dominant polycystic kidney disease is one of the most common human monogenic diseases. The disease affects between 1:1000 and 1:400 people and is characterized by massive enlargement of fluid-filled cysts of renal tubular origin that compromise normal renal parenchyma and eventually leads to renal failure (Torres and Harris, 2009; Zhou, 2009). Survival of the patients at end stage of polycystic kidney disease depends on lifelong hemodialysis or kidney transplantation. No alternative clinical treatment is currently available.

Researchers have been trying to elucidate the mechanisms that lead to cyst development and to determine drug targets for treating polycystic kidney disease. Cyst growth in polycystic kidney disease requires epithelial cell hyperplasia accompanied by fluid secretion into the cyst lumen (Wilson and Goilav, 2007). Multiple observations lead to the hypothesis that cyst cells are less-than-terminally differentiated, continue to proliferate, secrete fluid and destroy the surrounding normal tissue by expansion (Calvet and Grantham, 2001; Wilson, 2004). The cystic fluid was found to contain many hormonal activities, including antidiuretic hormone and epidermal growth factor, as well as a lipophilic substance, capable of stimulating the accumulation of cyclic adenosine monophosphate (cAMP) (Mangoo-Karim et al., 1989; Grantham et al., 1995; Yamaguchi et al., 1995). Both cystic epithelial cell proliferation and cystic fluid secretion are cAMP-dependent, which concerned with the signaling pathways (Torres, 2004). However, the detail mechanism of cystogenesis still remains unknown. Human polycystic kidney disease is mainly caused by mutations in one of two genes, *Pkd1* and *Pkd2*, encoding the interacting proteins polycystin-1 (PC1) and polycystin-2 (PC2), respectively (Harris and Torres, 2009). It has been reported that the mutant PC1 or PC2 causes renal cyst development by affecting multiple intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK) pathway, mammalian target of rapamycin (mTOR) pathway, Wnt pathway, etc. (Calvet, 2008; Pandey et al., 2008; Shibazaki et al., 2008). Therefore, hormones, hormone receptors, membrane transporters and intracellular signaling pathways involved in polycystic kidney disease have been identified as new potential therapeutic targets.

Recent studies have found that some chemical compounds have therapeutic roles on polycystic kidney disease. Gattone et al. (2003) and Torres et al. (2004) found that vasopressin-2-receptor antagonists are effective agents to reduce cyst volumes. Somatostatin

* Corresponding author. Department of Pharmacology, School of Basic Medical Sciences, Peking University, 38 Xueyuan Lu, Haidian district, Beijing 100191, China. Tel.: +86 10 82805622.
E-mail address: baoxue@bjmu.edu.cn (B. Yang).
[1] These authors contributed equally to the article.

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markedly inhibits chloride secretion in the shark rectal gland, suggesting inhibition of adenylcyclase and cAMP-dependent fluid secretion. Drugs that have shown beneficial effects in orthologous animal models of polycystic kidney disease include tolvaptan, src inhibitors, pioglitazone, etanercept and triptolide (Leuenroth et al., 2007, 2008; Patel et al., 2009), most of which inhibit the renal cyst development through regulating intracellular signaling pathways.

The purpose of this study is to identify the inhibitory effect of curcumin on renal cyst development and to investigate the inhibitory mechanism using *in vitro* cyst models. The results showed that curcumin significantly inhibited cyst formation and enlargement using a Madin–Darby canine kidney (MDCK) cell cyst model and an embryonic kidney cyst model. These data indicate that curcumin might be developed as a candidate therapeutic drug for polycystic kidney disease.

2. Methods and materials

2.1. Materials

Curcumin (Sigma) was dissolved in 100% DMSO to prepare a 100 mM stock solution and was stored at −20 °C. Anti-H-Ras (sc-35), anti-B-Raf (sc-166), anti-Raf-1 (sc-227), anti-p-MEK-1/2 (sc-7995), anti-p-ERK(sc-7383), anti-ERK2 (sc-153), anti-c-fos (sc-253), anti-Egr-1 (sc-110), anti-NAB2 (sc-22815), anti-β-actin (sc-1615-R), donkey anti-goat IgG (sc-2020) and goat anti-rabbit IgG (sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG and goat anti-rat IgG were purchased from Sigma.

2.2. MDCK cyst model

Type I MDCK cells (ATCC No. CCL-34) were cultured at 37 °C in a humidified 95% air/5% CO_2 atmosphere in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin. To generate cysts, four hundred MDCK cells were suspended in 0.4 ml of ice-cold Minimum Essential Medium containing 2.9 mg/ml collagen (PureCol, Inamed Biomaterials, Fremont, CA), 10 mM HEPES, 27 mM $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin (pH 7.4). The cell suspension was plated onto 24-well plates. After gelation for about 90 min, 1.5 ml of MDCK cell medium containing 10 μM forskolin was added to each well and plates were maintained at 37 °C in a 5% CO_2 humidified atmosphere. Forskolin, having a role in promoting the enlargement of cysts in autosomal dominant polycystic kidney disease (Putnam et al., 2007) caused MDCK cells to form cysts.

To identify cyst inhibitory activity, 10 μM curcumin was included in the culture medium in the continued presence of 10 μM forskolin from day 0 and onward. Medium containing forskolin and curcumin was changed every 12 h. On day 6, cysts (with diameters >50 μm) and non-cyst cell colonies were counted by phase-contrast light microscopy. Furthermore, to study the effect of curcumin on MDCK cyst enlargement, curcumin was added to medium in the continued presence of forskolin from day 4 after seeding and the medium containing forskolin and curcumin was changed every 12 h for 8 days. Micrographs showing the same cysts in collagen gels (identified by markings on plates) were obtained every 2 days. Cyst diameters were measured using Image-Pro Plus 6.0 to determine the growth rate of cysts. At least 10 cysts/well and 3 wells/group were measured for each condition.

2.3. MDCK tubule model

To determine if curcumin promoted MDCK cell to form tubule, MDCK cells were raised by 3T3 conditioned medium exposed to curcumin (at 0.4, 2 or 10 μM) for 12 days. The medium containing curcumin was changed every 12 h. On day 12, tubule numbers were counted by phase-contrast light microscopy. Furthermore, to determine if curcumin-induced MDCK cysts to form tubules, the established cysts (on day 4 cultured with forskolin) were raised by 3T3 conditioned medium containing different concentrations of curcumin over the next 8 days. Tubules were observed and photographed every 2 days during days 4–12 of culture. On day 12, the longest length of every tubule was determined by Image-Pro Plus 6.0.

2.4. Embryonic organ culture model

Mouse embryos were obtained at embryonic day 13.5 (E13.5) as reported in previous study (Tradtrantip et al., 2009). Metanephroi were dissected and placed on transparent Falcon 0.4 μm diameter porous cell culture inserts. 1:1 mixture of DMEM/Ham's F-12 nutrient medium is added in the lower chamber, supplemented with 2 mM L-glutamine, 10 mM HEPES, 5 μg/ml insulin, 5 μg/ml transferrin, 2.8 nM selenium, 25 ng/ml prostaglandin E, 32 pg/ml T3, 250 U/ml penicillin and 250 μg/ml streptomycin. Kidneys were maintained in a 37 °C humidified 5% CO_2 incubator for up to 6 days. Culture medium containing 100 μM 8-Br-cAMP, with or without curcumin, was replaced (in the lower chamber) every 12 h. Kidneys were photographed using a Nikon inverted microscope (Nikon TE 2000-S) equipped with 2× objective lens, 520 nm bandpass filter, and high-resolution PixeLINK color CCD camera. Cyst area was calculated by dividing the total cyst area by total kidney area.

2.5. Cytotoxicity, cell proliferation and apoptosis

MTT assay was used to assess cytotoxicity. MDCK cells (6400 cells/200 μl/well) in a 96-well plate were exposed to curcumin at 0, 0.1, 1, 10 or 100 μM for 24 h. 20 μl MTT solution (5 mg/ml) was added and incubated for 4 h at 37 °C. Then the medium was removed and 150 μl DMSO was added. The absorbance at 490 nm was measured. The cell viability was showed as OD_{490} value.

Cell proliferation was assayed using a cell counting kit-8 (CCK-8) kit at indicated time point (12 h, 24 h, 36 h, 48 h, 72 h and 96 h). MDCK cells (1000 cells/100 μl/well) in a 96-well plate were cultured in a 37 °C humidified 5% CO_2 incubator. Each well was added 10 μl CCK-8 solution and was incubated for 1 h. The absorbance at 450 nm was measured. Cell proliferation rate was expressed as OD_{450} value.

Apoptosis was measured using the *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN). MDCK cells were seeded on 8-chamber polystyrene tissue culture-treated glass slides and incubated with curcumin at 0.4, 2 or 10 μM. Gentamycin (2 mM) was used as positive control. Three days later, the assay was done according to manufacturer's instructions. Five microscopic fields were analyzed per condition. Apoptosis index was calculated as the percentage of nucleus-stained cells.

2.6. CFTR function assay

FRT epithelial cells stably coexpressing human CFTR and the high-sensitivity I^--sensing green fluorescent protein YFP-H148Q/I152L were used as described previously (Galietta et al., 2001). Cells were grown at 37 °C (90% humidity; 5% CO_2) for 24 h. At the time of the assay, cells were washed with PBS and then incubated with PBS containing forskolin (20 μM) and curcumin (at 0.1, 1 or 10 μM) for 20 min. Measurements were carried out using FLUOstar fluorescence plate readers (Optima; BMG LABTECH), each equipped with 500 ± 10 nm excitation and 535 ± 15 nm emission filters (Chroma Technology Corp.). Each well was assayed individually for I^- influx by recording fluorescence continuously (200 ms per point) for 2 s (baseline) and then for 12 s after rapid (<1 s) addition of 165 μl PBS in which 137 mM Cl^- was replaced by I^-. I^- influx rate was computed by fitting the final 11.5 s of the data to an exponential for extrapolation of initial slope and normalizing for background-subtracted initial fluorescence. All

experiments contained negative control (DMSO vehicle) and positive control $CFTR_{inh}172$.

2.7. Short-circuit current measurements

MDCK cells in Snapwell inserts (transepithelial resistance 1000–2000 Ω) were cultured in medium containing curcumin (at 0.1, 1 or 10 μM) for 1 or 48 h. Curcumin was washed out with medium for 1 h before short-circuit current measurements. Snapwell inserts containing MDCK cells were mounted on a standard Ussing chamber system. Permeabilization treatment with 250 μg/ml amphotericin B was added on the basolateral membrane of the insert. The hemichambers were filled with 5 ml of 65 mM NaCl, 65 mM Na-gluconate, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, Na-Hepes and 10 mM glucose (apical), and 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, Na-Hepes and 10 mM glucose (basolateral) (pH 7.3). Short-circuit current was recorded continuously using a DVC-1000 voltage clamp (World Precision Instruments, Sarasota, FL) with Ag/AgCl electrodes and 1 M KCl agar bridges.

2.8. cAMP measurement

MDCK cells cultured in six-well plates were exposed to curcumin (at 0.4, 2 or 10 μM) for 30 min with or without 10 μM forskolin stimulation. Intracellular cAMP content was determined by RIA following the procedure recommended in the cAMP RIA kit (Chinese People's Liberation Army General Hospital, Beijing). The result was recorded by an SN-695 gamma counter RIA program Ver 6.0.

2.9. Western blotting

MDCK cells were seeded in six-well plates in appropriate medium containing 10% FBS for 2 h, followed by serum starvation in serum-free 1:1 mixture of DMEM and Ham's F-12 nutrient medium for 24 h. Standard protocol involved exposure to curcumin (at 0.4, 2 or 10 μM) for 1 h with or without 10 μM forskolin stimulation. Western blot analysis was performed as described in manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA).

2.10. Statistical analysis

Values were expressed as mean ± S.D. Statistical analysis was performed by one way ANOVA. A level of $P<0.05$ was considered to be significant.

3. Results

3.1. Curcumin inhibits MDCK cyst formation and enlargement

An MDCK cyst model was used to identify the cyst inhibitory effect of curcumin. MDCK cells did not form cysts, but cells grew into colonies, in the absence of forskolin (Fig. 1A, *left*). Cysts were seen in 3 to 4 days and progressively expanded over the next 8 days (Fig. 1A, *middle*) in the presence of 10 μM forskolin. To determine cyst inhibitory activity, MDCK cells were incubated from day 0 to day 6 with curcumin in the presence of 10 μM forskolin. On day 6, spherical cysts (with diameter >50 μm) and non-cyst cell colonies were counted in each well. Interestingly, curcumin significantly inhibited cyst formation (Fig. 1A *right*, Fig. 1B) up to 62% with dose–response relationship. However the numbers of total colonies (cysts plus non-cyst colonies) were similar with the original seeded cell numbers in curcumin-treated wells, which indicates that curcumin did not destroy MDCK cells. The inhibitory activity of curcumin on cyst formation (IC_{50} was 0.12 μM) is showed in Fig. 1C.

To determine the effect of curcumin on cyst enlargement, the established cysts (on day 4 cultured with forskolin) were exposed to 0.4, 2 or 10 μM curcumin in the presence of 10 μM forskolin. Cysts were observed and photographed every 2 days from day 4 to day 12 of culture (Fig. 2A). Cyst continuously enlarged with forskolin stimulation (Fig. 2A *top panel*). Curcumin significantly inhibited cyst enlargement (Fig. 2A *middle panel* and Fig. 2B). Cyst enlargement inhibition was reversible when curcumin was washed out after 4 day treatment (Fig. 2A, *bottom panel*, Fig. 2C).

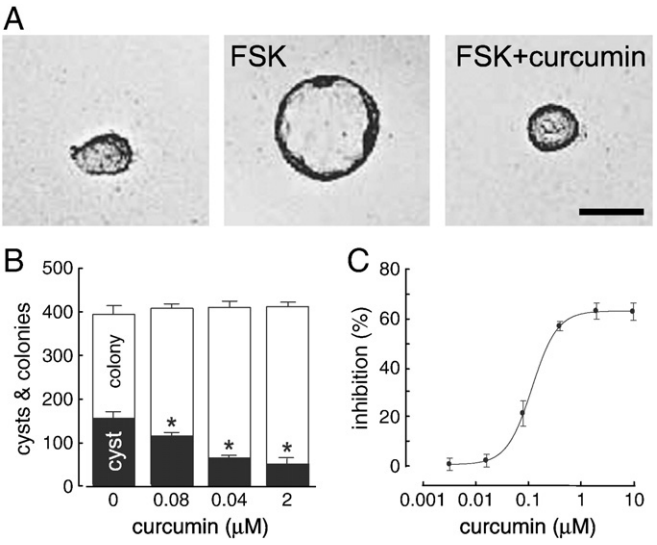

Fig. 1. Curcumin inhibits MDCK cyst formation. (A) Representative light micrographs of MDCK cell culture in collagen gels. Light micrographs were taken on day 6 after cell seeding. MDCK cells were cultured continuously without (*left*) or with 10 μM forskolin (FSK, *middle*) or 10 μM forskolin plus 2 μM curcumin (*right*). Scale bar, 50 μm. (B) MDCK cyst formation rate. White bars show the total numbers of colonies (including cysts and non-cyst colonies) per well on day 6 after MDCK cell being cultured with 10 μM forskolin in the absence (control) and presence of curcumin at indicated concentrations. Black bars show the numbers of cysts with diameter >50 μm (mean ± S.D., n = 3, $^*P<0.05$ vs. control). (C) Inhibition activity–concentration profile of curcumin on MDCK cyst formation.

3.2. Effect of curcumin on biological characteristics of MDCK cells

To test whether the cyst inhibition was caused by cytotoxicity of curcumin on MDCK cells, MTT assay was used to determine cell viability. At <100 μM, curcumin did not reduce MDCK cell viability as showed as OD_{490} in Fig. 3A. Furthermore, at concentrations lower than 10 μM, curcumin did not induce MDCK cell apoptosis analyzed by TUNEL Assay Kit. Gentamicin (2 mM) as positive control caused significantly MDCK cell apoptosis (Fig. 3B). At normal cell culture condition, MDCK cell proliferation rate was not significantly affected by curcumin treatment at the doses inhibiting cyst enlargement detected by CCK-8 assay (showed as OD_{450} in Fig. 3C). However, curcumin inhibited forskolin-increased MDCK cell proliferation rate (Fig. 3D). All these results indicate that curcumin inhibits cyst development neither by cytotoxicity nor by apoptosis in MDCK cells, but by inhibiting forskolin-stimulated MDCK cell proliferation.

MDCK tubule model was used to define whether curcumin inhibit cyst development by promoting the differentiation of cyst epithelial cells. Both MDCK cells and cysts formed renal tubule-like structures with 3T3 conditioned medium incubation, (Fig. 4A *middle*). When MDCK cells were incubated with curcumin in the presence of 3T3 conditioned medium for 12 days, the numbers of MDCK cells forming tubule-like structures increased with doses of curcumin (Fig. 4B). After the established MDCK cysts were incubated with or without curcumin in the presence of 3T3 conditioned medium for 8 days, average lengths of the longest tubule from each MDCK cyst were about 3 fold longer in 10 μM curcumin-treated group than those in control group that was cultured with only 3T3 conditioned medium (Fig. 4A *right*, Fig. 4C). The data suggest that curcumin promotes MDCK cell differentiation.

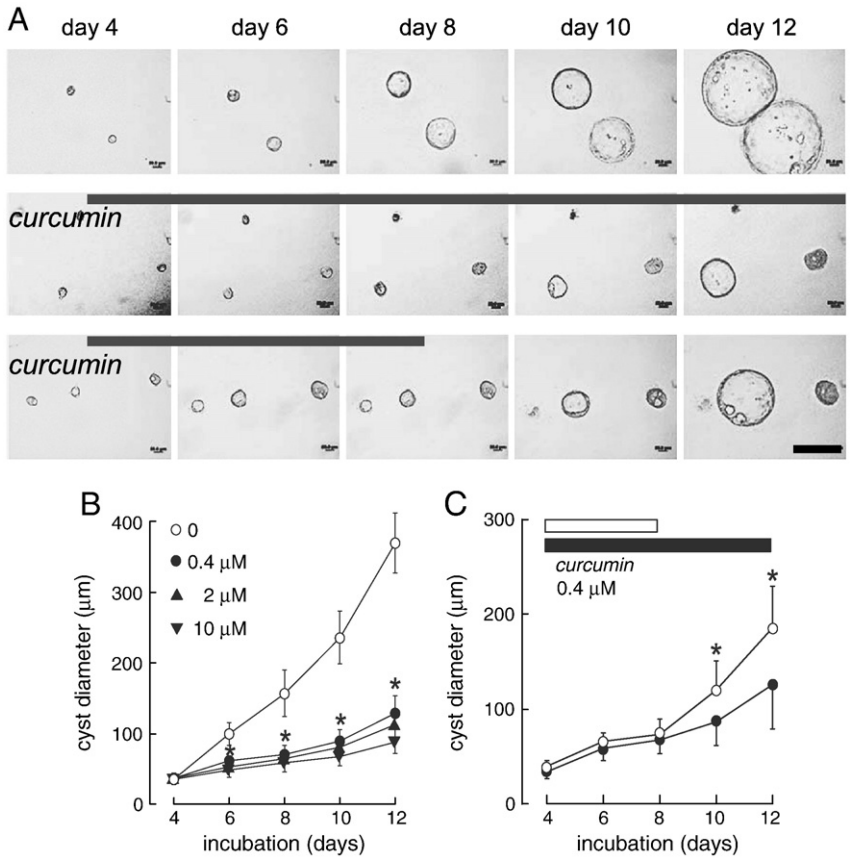

Fig. 2. Curcumin slows MDCK cell cyst enlargement. (A) Representative light micrographs of MDCK cell cyst enlargement in collagen gels. Light micrographs taken at indicated days after cell seeding. MDCK cells were exposed continuously to 10 μM forskolin (top panel). In some experiments, curcumin was added for 8 days (middle panel) or 4 days (bottom panel), from day 4 onward after cell seeding in gels. Scale bar, 200 μm. Thick lines indicate the culture time with curcumin. (B) MDCK cell cyst enlargement shown as cyst diameters for curcumin dose–response (mean ± S.D., >30 cysts analyzed per time point, $^*P<0.05$ vs. control). (C) Inhibition was reversible as shown by exposure to curcumin at days 4–8 followed by washout (mean ± S.D., >30 cysts analyzed per time point,$^*P<0.05$ vs. curcumin-treated for 8 days group). White line and dots represent 4 days curcumin treatment; black line and dots represent 8 days curcumin treatment.

Due to the cyst liquid secretion depends on CFTR function in cyst epithelia (Yang et al., 2008). To determine if the inhibitory activity of curcumin on cyst formation and enlargement was related to the gating or expression regulation of CFTR, CFTR function was determined in FRT cells expressing CFTR by an I^- sensitive fluorescence assay and in MDCK cells by a Ussing chamber assay. There was no difference in CFTR-mediated I^- secretion between curcumin-treated and untreated FRT cells (Fig. 5A). Fig. 5B shows that curcumin did not inhibit the short-circuit current in MDCK cells following the stimulation by forskolin. Curcumin did not alter CFTR expression as seen by short-circuit current in MDCK cells after 1 verses 48 h incubation with 0.1, 1, 10 μM curcumin followed by washout. The representative curves are showed in Fig. 5C. These results indicate that curcumin does not affect the CFTR-mediated cyst liquid secretion.

3.3. Curcumin regulates intracellular signaling pathways

Fig. 6 shows the cAMP level in MDCK cells incubated with forskolin in the presence or absence of curcumin. Intracellular cAMP content was significantly increased in MDCK cells incubated with forskolin. There was no difference in intracellular cAMP concentration between curcumin-treated and untreated MDCK cells exposed to forskolin.

The proteins involved in intracellular signaling pathway were analyzed by Western blotting. The highest p-ERK expression level in serum-starved MDCK cells was found at the 60th min in the presence of 10 μM forskolin (Fig. 7A). Based on this time course, serum-starved MDCK cells were treated with curcumin at 0.4, 2 and 10 μM for 60 min in the presence of 10 μM forskolin. Curcumin significantly decreased levels of Ras, B-raf, p-MEK and p-ERK, and increased Raf-1 level in MDCK cells stimulated with 10 μM forskolin. c-fos expression was significantly down-regulated by curcumin. With curcumin treatment, Egr-1 was down-regulated and NAB-2 was up-regulated (Fig. 7B and C).

3.4. Curcumin retards cyst development in an embryonic kidney organ culture model

To further evaluate the effect of curcumin on renal cyst formation and enlargement in whole organ condition, the embryonic kidneys from wild-type mice at embryonic day 13.5 (E13.5) were cultured for 4 days in the absence or presence of 100 μM 8-Br-cAMP. In the absence of 8-Br-cAMP, kidneys increased in size over 4 days, whereas numerous cystic structures were seen in the presence of 8-Br-cAMP (Fig. 8A, *top panel*). Curcumin significantly reduced cyst formation and enlargement (Fig. 8A, *middle panel*). Cysts formed and enlarged again following curcumin washout after two-day treatment, which indicate that the cyst inhibition caused by curcumin was reversible (Fig. 8A, *bottom panel*). The effect of curcumin on cyst formation and enlargement was dose-dependent (Fig. 8B), as confirmed by quantitative image analysis (Fig. 8C). Moreover, kidney growth with or without 8-Br-cAMP incubation was not affected by curcumin (data not shown).

4. Discussion

The motivation of this study is to identify the effect of curcumin on cyst development in polycystic kidney disease. Using MDCK cyst

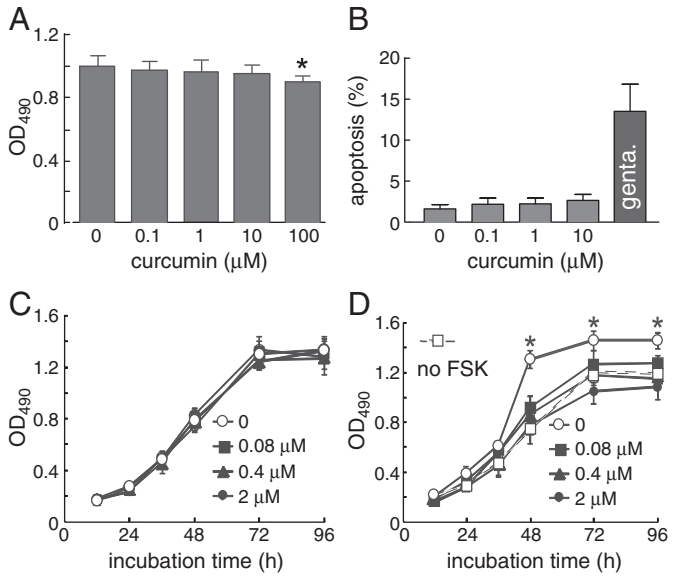

Fig. 3. Effect of curcumin on MDCK cell biological properties. (A) Cytotoxicity assayed by MTT assay (mean ± S.D., n = 3, *P<0.05 vs. control). MDCK cells were cultured in the absence or presence of curcumin for 24 h. Cell viability is showed as OD_{490}. (B) MDCK cell apoptosis assayed by the detection of fluorescein-dUTP-labeled DNA strand breaks by fluorescence microscopy after culturing with curcumin for 72 h. DMSO was used as negative control. Gentamicin (genta. 2 mM) was used as positive control. (C) Effect of curcumin on MDCK cell proliferation measured by CCK-8 kit and showed as OD_{450} (mean ± S.D., n = 3). Curcumin was present in the medium at indicated concentrations. DMSO was used as negative control. (D) Effect of curcumin on forskolin-stimulated MDCK cell proliferation measured as described above. Curcumin was present in the medium at indicated concentrations with 10 μM forskolin (FSK). DMSO was used as negative control (without forskolin and curcumin) expressed with white squares and dash line (mean ± S.D., n = 3, *P<0.05 vs. curcumin-treated groups).

model, we found that curcumin significantly inhibited cyst formation and enlargement. The cyst inhibition activity of curcumin was also confirmed in an embryonic kidney cyst model. In normal condition,

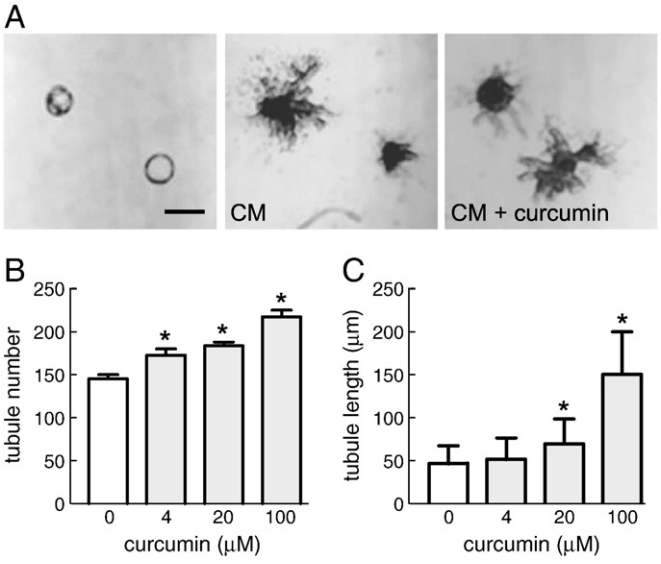

Fig. 4. Effect of curcumin on the tubulogenesis in MDCK cells and MDCK cysts. (A) Representative light micrographs of tubule-like structures on the MDCK cysts in collagen gels. Light micrographs taken on day 12 after MDCK cells in collagen gel exposed continuously to 10 μM forskolin for 4 days and 3T3 conditioned media (CM) without or with curcumin at 10 μM over the next 8 days. Scale bar, 50 μm. (B) The numbers of cells forming tubule-like structures without or with curcumin treatment (mean ± S.D., n = 3, *P<0.05 vs. control). (C) The average values of the longest length of tubule-like structures on each MDCK cyst without or with curcumin treatment (mean ± S.D., > 30 tubules analyzed, *P<0.05 vs. control).

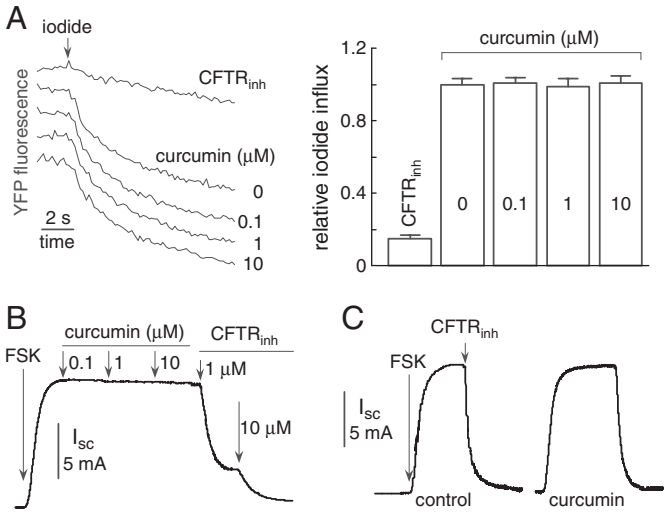

Fig. 5. Effect of curcumin on the function and expression of CFTR. (A) Representative original fluorescence data from individual wells showing I^- influx induced by adding an I^--containing solution after addition of CFTR inhibitor ($CFTR_{inh}$ or curcumin (*left panel*). *Right panel* shows averaged I^- influx for experiments done as in left panel (mean ± S.D., n = 3). (B) Effect of curcumin on short-circuit current in MDCK cell monolayer stimulated by 20 μM forskolin (FSK). (C) Short-circuit current in MDCK cell monolayers cultured without or with 10 μM curcumin for 1 or 48 h. Curcumin was washed out for 1 h before measurements. CFTR chloride current was stimulated by 20 μM forskolin.

curcumin at <100 μM had no cytotoxicity in MDCK cells and embryonic kidneys.

Curcumin (diferuloylmethane), a polyphenol natural product isolated from the rhizome of the plant *Curcuma longa*, has garnered great interest in recent years as a potential therapeutic agent for the treatment and/or prevention of various disease processes, including neurodegenerative disorders (Begum et al., 2008), inflammatory-related diseases (Jurenka, 2009), fibrosis diseases (Lipecka et al., 2006; O'Connell and Rushworth, 2008) and cancers (Deeb et al., 2007). To our knowledge, this is the first study that defines a cyst inhibition activity of curcumin and provides a rationale for developing curcumin as a candidate drug for polycystic kidney disease.

Multiple intracellular factors, including hormones, hormone receptors and membrane transporters, are involved in cystogenesis and cyst enlargement in polycystic kidney disease. *In vitro* cyst models provide useful tools for defining these factors as drug targets and evaluate candidate drugs for polycystic kidney disease. MDCK cyst model has been used for screening candidate inhibitors of cyst formation and enlargement and exploring the inhibitory mechanisms (Taide et al., 1996). In this model, MDCK cell cultured in three-dimensional collagen gels generate polarized, single-layer, thinned epithelium surrounding fluid-filled space (Yang et al., 2008). MDCK cells in cysts undergo proliferation, fluid transport and matrix remodeling, as seen in tubular epithelial cells cultured from kidneys of polycystic kidney disease. Cyst formation and enlargement depend

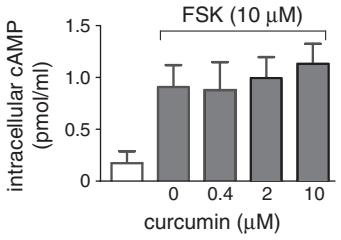

Fig. 6. Effect of curcumin on intracellular cAMP in MDCK cells. Intracellular cAMP concentration was measured by cAMP RIA kit. MDCK cells were exposed to 10 μM forskolin (FSK) without or with curcumin treatment for 30 min. Data are mean ± S.D., n = 6.

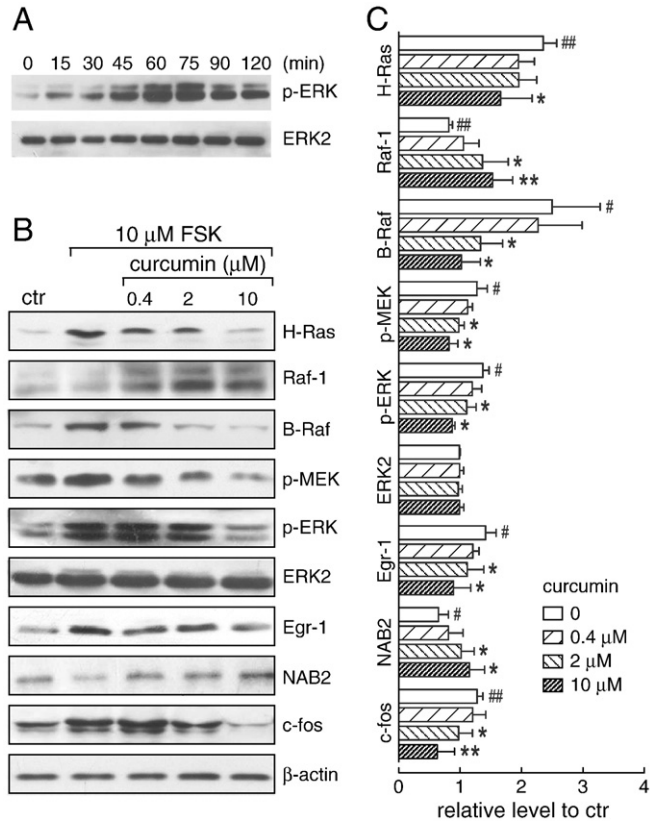

Fig. 7. Curcumin regulates intracellular pathways in MDCK cells. (A) The time course of the p-ERK level in MDCK cells with forskolin incubation detected with Western blotting analysis. (B) Representative Western blots of signaling proteins from MDCK cells cultured with curcumin at indicated concentrations in the presence of 10 μM forskolin (FSK) for 60 min. Control (ctr) means the sample from MDCK cells without curcumin and forskolin treatment. Each lane was loaded 20 μg protein. (C) The quantitative analysis of signaling protein expression in MDCK cells as described above. Relative level means the ratio of Western blotting band density in testing groups (with forskolin or forskolin plus curcumin) to that in control group. Data are mean ± S.D., n = 6; #P<0.05 ##P<0.01 vs. control, *P<0.05, **P<0.01 vs. forskolin treated group.

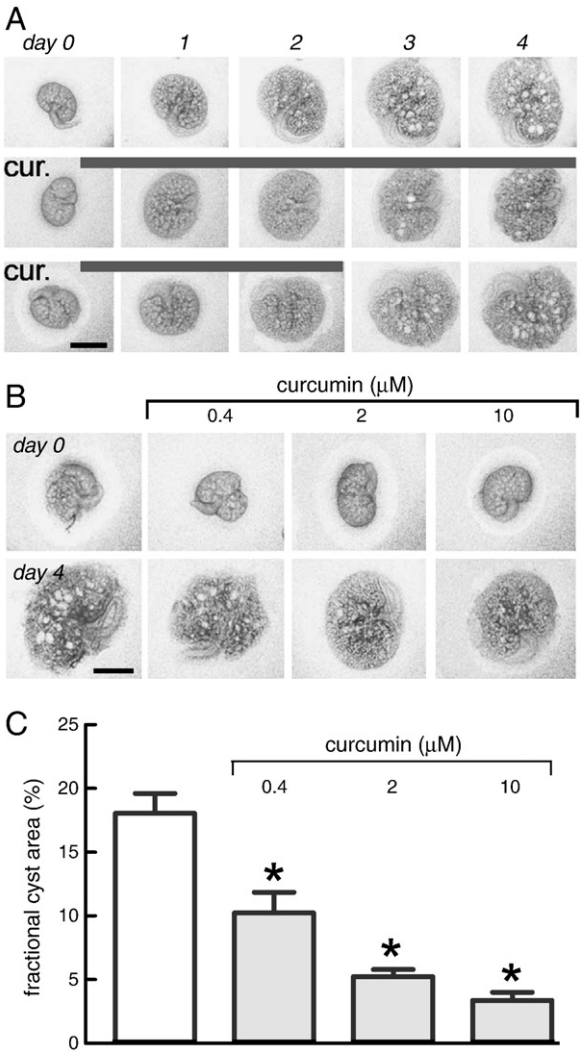

Fig. 8. Curcumin slows cyst growth in embryonic kidney organ cultures. Embryonic kidneys at day E13.5 were placed in culture and maintained for 4 days. (A) Kidney appearance by transmitted light microscopy for cultures in the continued presence (top panel) of 100 μM 8-Br-cAMP, continued presence (middle row) of 100 μM 8-Br-cAMP and 2 μM curcumin (cur.) or continued presence (bottom row) of 100 μM 8-Br-cAMP and only two days 2 μM curcumin. Each series of photographs shows the same kidney on successive days in culture. Scale bar, 1 mm. Thick lines indicate the culture time with curcumin. (B) Inhibition of cAMP-induced cyst growth by curcumin at indicated concentration. Images show embryonic kidneys before (day 0) and after (day 4) continuous curcumin treatment. Scale bar, 1 mm. (C) Fractional cyst area in control and curcumin-treated kidneys (mean ± S.D., n = 6–10, *P<0.05 vs. control).

on cAMP, which is thought to increase cell proliferation and activate CFTR-facilitated transepithelial fluid secretion (Torres, 2008). In this study, a series of experiments with MDCK cells were performed to explore the mechanism by which curcumin inhibited cyst formation and enlargement.

Cyst development in polycystic kidney disease requires epithelial cell hyperplasia (Calvet, 2008). To determine whether curcumin inhibited cyst enlargement directly from MDCK cell cytotoxicity, we detected the effect of curcumin on MDCK cells incubated under normal culture condition or with forskolin stimulation. Our experimental results excluded the cyst inhibition from cytotoxicity, low cell viability and cell apoptosis. However, curcumin inhibited forskolin-stimulated MDCK cell proliferation, which indicates that abnormal proliferation of MDCK may be an initial factor in cystogenesis. The results from embryonic kidney culture model showed that curcumin reversibly inhibited cyst formation and enlargement in embryonic kidneys but did not affect intact kidney growth, which was consistent with the results from MDCK cyst model.

Multiple observations suggest that cyst cells are less-than-terminally differentiated, continue to proliferate (Calvet and Grantham, 2001; Wilson, 2004). The MDCK tubule formation experiments, a model for evaluating differentiation activity in MDCK cells (Pollack et al., 1998; Jiang et al., 2000; Battini et al., 2006), showed that the curcumin significantly promoted tubule-like structure formation and prolongation. The results suggest that cyst inhibitory activity of curcumin may be relative to epithelial cell differentiation.

Previous studies suggest that the renal cyst enlargement depends on CFTR-mediated cystic fluid secretion (Calvet and Grantham, 2001; Wilson, 2004). CFTR is a drug target for inhibiting polycystic kidney disease progressing (Yang et al., 2008). The effects of curcumin on the functional activity and expression regulation of CFTR were detected in this study. The data showed that curcumin did not change CFTR expression level and forskolin induced CFTR gating. However, it is not excluded that curcumin affects other membrane transporters involved in fluid secretion.

In polycystic kidney disease, previous evidence has demonstrated that cAMP drives both abnormal cystic epithelial cell proliferation by stimulating MAPK pathway (Calvet and Grantham, 2001; Wilson, 2004) and fluid secretion into the cyst lumen by activating CFTR chloride channel (Cowley, 2008). We measured the cAMP level in MDCK cells. Forskolin significantly increased the intracellular cAMP concentration. However, curcumin did not reduce the raised cAMP levels. The result

indicates that the target of curcumin on cyst inhibition may be at downstream of cAMP signaling, but not at upstream.

It has been described that Ras/MAPK pathway is an effective pathway in cAMP-stimulated cell proliferation in human polycystic kidney cells in culture (Yamaguchi et al., 2003). Previous studies have shown that the opposite rate of B-Raf/Raf-1 may result in the phenotypic difference between normal kidney cells and polycystic kidney cells exposed to cAMP (Yamaguchi et al., 2004). The extracellular signal-regulated kinases 1/2 (ERK1/2) are inhibited in normal cells treated with cAMP due to a cAMP-dependent inhibitory phosphorylation of Raf-1. In contrast, cAMP treatment of polycystic kidney cells activates ERK signal, which is induced by cAMP-dependent activation of B-Raf, not seen in normal cells (Yamaguchi et al., 2003, 2006). We examined the effect of curcumin on Ras/MAPK pathway by detecting the expression and/or phosphorylation of proteins Ras, Raf, MEK and ERK, which are downstream of cAMP. The results showed that curcumin regulated the rate of B-Raf/Raf-1 by mainly down-regulating the levels of B-Raf and up-regulating the levels of Raf-1, which indicates that curcumin reduced levels of p-MEK and p-ERK directly or through Ras and B-Raf regulation. Therefore, curcumin may inhibit the cystic cell hyperplasia by reversing the increased rate of B-Raf/Raf-1 in MDCK cyst model.

The downstream of Ras/B-Raf/MEK/ERK pathway includes c-fos, Egr-1 and NAB2 (Mayer et al., 2009). It has been reported that c-fos dimerises with c-jun to form the AP-1 transcription factor, which upregulates transcription of a diverse range of genes involved in proliferation and differentiation in polycystic kidney disease (Le et al., 2005). As a key regulator of cellular proliferation, apoptosis and mediator of inflammation, Egr-1 is consistently implicated in various pathophysiological processes. Egr-1 expression was mediated by the MAPK pathway. Pharmacological inhibition of ERK-1/2 activation abolished Egr-1 expression (Hasan and Schafer, 2008). To confirm the effect of curcumin on MAPK pathway, the downstream kinases of MAPK pathway were also examined in this study. The results showed that Egr-1 was down-regulated and NAB2 was up-regulated by curcumin, which support that Ras/MAPK pathway is involved in cyst inhibition caused by curcumin. Furthermore, curcumin significantly inhibited c-fos. All these data suggest that the Ras/MAPK pathway regulation could be a key mechanism of curcumin mediated MDCK cyst inhibition. However, other signaling pathways, such as mTOR/p70S6K and Wnt pathways (Shinojima et al., 2007; Covic, 2008; Yu et al., 2008) are still not excluded in MDCK cyst development based on our study.

In summary, the data here indicate that curcumin, at concentrations without apparent toxicity, retarded the development of renal cysts in *in vitro* models by inhibiting cyst epithelia proliferation and promoting epithelia differentiation. The mechanism of cyst inhibition may be involved in curcumin regulated Ras/MAPK signaling pathway. The further *in vivo* studies should be done in polycystic kidney disease mouse model to define that curcumin as a therapeutic agent for polycystic kidney disease.

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